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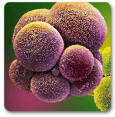
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Effect of cytochalasin types on the production of heterozygous parthenogenetic porcine embryos and the isolation of putative parthenogenetic embryonic stem cells

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Abstract

Parthenogenetic embryos have been suggested as an alternative source of embryonic stem cells (ESCs). The present study was undertaken to determine the efficiency with which porcine heterozygous parthenotes could be produced using cytochalasin B (CB) or cytochalasin D (CD) and whether parthenogenetic ESCs (pESCs) could be isolated from these. Cleavage rate was lower and fewer embryos developed to the blastocyst stage in the CB group compared with the CD group. The number of primary outgrowths obtained was also lower in the CB compared with the CD group. No primary lines were isolated from embryonal outgrowths in the CB group. In contrast, primary cell lines were derived from these in the CD group. These lines survived vitrification and warming, resulting in established cell lines, which maintained a characteristic ESC morphology and expressed the pluripotent markers Oct4 and Nanog following repeated passaging. Putative pESC lines could also be directly differentiated to cell types representative of all three germ layers.

Keywords: Parthenotes, porcine, heterozygosity, heterozygous ESC

Introduction

Embryonic stem cells (ESCs) are pluripotent cells derived from epiblast of blastocysts [1,2]. These cells have the capacity for unlimited self-renewal and can be directed to differentiate into all cell types in the body. As such, ESCs are thought to hold considerable potential for treating a range of degenerative diseases and injuries [3]. However, the isolation of ESCs from human embryos raises religious, social, and ethical issues because it involves the destruction of embryos, which has limited research in this area. As such, alternative sources of ESCs are being examined to overcome these issues, including the isolation of ESCs from parthenogenetic embryos. Parthenogenetic ESCs (pESCs) have been isolated for various mammalian species, but have only been extensively characterized in mice [4-6]. This has included directed differentiation into cell types representative of all three germ layers and the production of chimeric mice and the demonstration of germline transmission [6,7].

Mammalian parthenogenetic embryos (parthenotes) are produced by the artificial activation of oocytes. These are normally diploidized using a range of chemicals to inhibit polar body extrusion. Depending on when polar body extrusion is inhibited, two types of diploid parthenotes can be produced. The first polar body contains homologous chromosomes while second polar body contain sister chromatids. When meiosis is blocked at metaphase I the oocyte remains tetraploid ($4n$), homologous chromosomes remain unseparated, the first

meiotic division does not occur and the first polar body is not extruded. Following activation and the resumption of meiosis the second polar body containing sister chromatids is extruded. These diploid parthenotes are heterozygous because they contain maternal and paternal (biparental) alleles. In contrast, during the inhibition of polar body extrusion after activation at metaphase II the sister chromatids remain unseparated and as result the homozygous parthenotes are produced (uniparental genome).

While homozygous parthenotes have been produced for a range of species, heterozygous parthenotes have only been produced in mice and pigs [5,8,9]. With that heterozygous parthenotes derived from the oocytes of the same donor are practically a clones of donor of oocytes because have the same genome as donor of oocytes but with different gene arrangement because of cross over [9]. While a range of diploidization agents have been reported the majority of studies have used cytochalasin B (CB) to inhibit polar body extrusion. The cytochalasins are cell-permeable toxins, which cause actin filament disruption and inhibit actin polymerization preventing the resumption of meiosis and polar body extrusion. However, CB has been shown previously to be cytotoxic. In the pig oocytes are exposed to CB for a relatively long time (22h) in order to produce heterozygous parthenotes which may a detrimental effect on the viability of parthenotes which in turn may affect the ability to isolate ESCs from these [8]. In contrast,

CD is thought to be less cytotoxic [10].

While homozygous parthenogenetic ESC lines have been isolated for a range of species, ESC lines from heterozygous embryos have been isolated in mice only [5]. The aim of the present study therefore was to determine the efficiency with which CB and CD could be used to generate heterozygous parthenotes in pigs and whether pESCs could be isolated from these using a method developed previously by us for the isolation of porcine ESCs from *in vitro* produced and somatic cell nuclear transfer (SCNT) embryos [11-13].

Materials and methods

All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

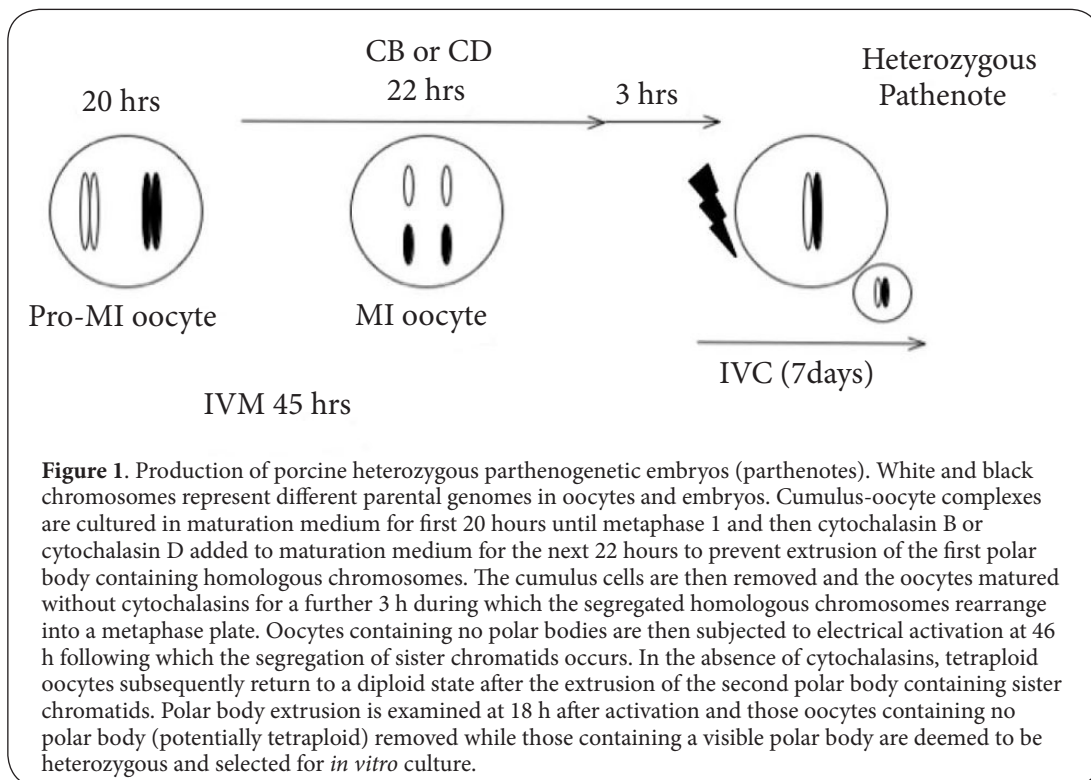
Oocyte collection and maturation

Sow oocytes were collected and matured *in vitro* as described previously by Beebe et al., [14] with minor modifications. Ovaries were transported from a local abattoir to the laboratory in 0.9% sodium chloride solution at between 33°C and 37°C. Cumulus-oocyte complexes (COCs) were matured for 46 h in BOMED medium [15] under mineral oil in a humidified

atmosphere of 5% CO₂, 5% O₂ and 90% N₂ at 38.5°C. BOMED medium consisted of Medium 199 (Life technologies, USA) supplemented with 0.5 mM cysteamine, 0.1 mg/ml sodium pyruvate, 75 µg/ml penicillin G, 50 µg/ml streptomycin sulphate, 5 µg/ml insulin, 5 µg/ml follicle stimulating hormone (FSH), 10 ng/ml recombinant human epidermal growth factor (EGF) and 10% sow follicular fluid.

Heterozygous parthenotes production

Heterozygous parthenotes were produced as described previously with minor modifications [8] by treating *in vitro* maturing oocytes with 5 µg/ml CB or 2.5 µg/ml CD at 20h of maturation for 22 hours (Figure 1). Oocytes were freed of cumulus cells by brief exposure to 0.1% hyaluronidase and manual pipetting and were activated 3 hours after treatment as previously described [16]. Before activation, oocytes were examined and those with polar bodies were excluded from experiment to avoid of the inclusion of homozygous haploid parthenotes. Denuded oocytes were activated by two 60-µsec 150V/mm direct current pulses with 1 sec apart using the Electro Cell Manipulator 2001 (BTX). Activated oocytes were cultured overnight and examined again for the presence of



polar bodies. Oocytes without polar bodies were excluded from experiment to avoid the inclusion of tetraploid parthenotes.

In vitro embryo culture

Parthenogenetic embryo culture was conducted in a two-

stage system as described previously by Beebe et al., [16]. Briefly, embryos were cultured in 50-µl droplets of modified NCSU23 from days 0 to 3 and in 45-µl droplets of standard NCSU23 [17] from days 4 to 7 under mineral oil in a humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂ at 38.5°C. The modified

NCSU23 (NCSU23-plg-neaa) contained 0.2 mM pyruvate, 5.7 mM lactate, 0.6 mM glucose and 1% MEM non-essential amino acids (Life technologies, USA) whereas the standard NCSU23 (NCSU23-neaa-eaa) contained 5.6 mM glucose, 1% MEM non-essential and essential amino acids (Life technologies, USA). Cleavage rate was assessed on day 3 when the culture medium was changed, and only embryos with more than 2 cells were cultured further. Ten percent heat inactivated foetal bovine serum (FBS) was added on early day 5 and blastocyst rate was assessed on day 7. Only expanded or hatched blastocysts with a defined inner cell mass and thin zona pellucida were selected for pESC isolation.

Parthenogenetic embryonic stem cell isolation

pESC were isolated from parthenotes as described previously [11-13]. Day-7 blastocysts with zona pellucida removed were gently pressed onto mitotically inactivated MEF feeder layers using a 30-gauge syringe and cultured in porcine ESC isolation medium for 6-12 days to obtain embryonal outgrowths. The isolation medium consisted of α MEM medium with GlutaMAX-I (Life technologies, USA) supplemented with 10% KnockOut serum replacement (SR), 1% MEM non-essential amino acids, 1% sodium pyruvate, 1% insulin-transferrin-selenium (ITS), 55 μ M 2-mercaptoethanol, 10 ng/ml recombinant human basic fibroblast growth factor (bFGF), 10 ng/ml recombinant human EGF (all from Life Technologies, USA), 10 ng/ml recombinant human Activin A (R&D Systems, USA), 10 ng/ml recombinant human leukemia inhibitory factor (LIF) and 0.5% antibiotic-antimycotic solution. Culture was conducted in a humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂ at 38.5°C. Medium was changed every 4-6 days. Putative porcine pESCs were identified according to morphological criteria described previously [11-13] including polygonal shape, 10-15 μ m in diameter, a small cytoplasmic/nuclear ratio, multiple lipid inclusions in the cytoplasm and a single nucleus with multiple nucleoli. Homogenous embryonal outgrowths, which had reached 3-5 mm in diameter, were cut into 200 μ m square pieces and re-plated onto fresh feeder layer to passage the cells. Expanded pESC colonies were passaged repeatedly and cultured as above.

Cleavage rate, blastocyst formation rate, primary embryonal outgrowth formation rate and the efficiency with which primary pESC lines could be isolated were analyzed by Fisher's exact test with 95% confidence interval using Graph Pad Prism 5 software (Graph Pad Software, USA).

Vitrification and warming

Porcine pESC lines were considered viable if they survived cryopreservation. pESCs were vitrified and warmed as previously described [11]. Briefly, pESC colonies were cut into pieces and vitrified using the CryoLogic Vitrification Method (CVM; CryoLogic Pty. Ltd). pESC colony pieces were held in 1 ml of the base medium containing α MEM medium with GlutaMAX-I (Life technologies, USA) supplemented with 25

mM HEPES buffer solution (Life technologies, USA) and 20% FBS. 8-10 pieces at a time were initially washed in fresh base medium, then in 1 ml of the base medium supplemented with 10% dimethyl sulfoxide (DMSO) and 10% ethylene glycol for 1 min and in 1 ml of the base medium supplemented with 20% DMSO, 20% ethylene glycol and 0.3 M sucrose (vitrification medium) for 20 sec, loaded in an approximately 3 μ l droplet of the vitrification medium onto a nylon hook and eventually vitrified by touching the hook onto the surface of a metal block which had been cooled by liquid nitrogen. The nylon hooks were then covered with cooled plastic sleeves, plunged into liquid nitrogen and stored. For warming pESC colony pieces, the vitrified droplets were dissolved and washed in 1 ml of the base medium supplemented with 0.2 M sucrose for 1 min. The colony pieces were then washed once in 1 ml of the base medium supplemented with 0.1 M sucrose and twice in 1 ml of the base medium, with 5 min for each wash. The warmed pieces were plated onto fresh feeder layer.

Immunofluorescence for co-expression of pluripotent markers

pESC colonies were fixed and permeabilized with 4% paraformaldehyde (PFA) and 0.3% saponin (Calbiochem, Germany) for 30 min, washed 3 times with DPBS (Life technologies, USA) supplemented with 0.3% saponin, blocked with DPBS supplemented with 0.3% saponin and 10% normal donkey serum (blocking solution) for 30 min, incubated with primary antibodies diluted in blocking solution overnight at 4°C, washed 3 times, incubated with secondary antibodies diluted in blocking solution for 1 h in the dark, washed 3 times and mounted with Slow Fade Gold antifade reagent with DAPI (Life technologies, USA). The primary antibodies were goat anti-Oct3/4 (1:100 dilution; sc-8628, Santa Cruz Biotechnology) and rabbit anti-Nanog (1:200 dilution; Merk-Millipore, Germany). The secondary antibodies were donkey anti-goat IgG-FITC (1:200 dilution; sc-2024, Santa Cruz Biotechnology) and goat anti-rabbit Ig-TXRD (1:200 dilution; Southern Biotech, USA). MEFs were used as negative control. Fluorescent images were captured using Leica TCS SP5 confocal laser scanning microscope (Leica Microsystem, Germany).

In vitro differentiation

Heterozygous pESC lines were further examined in terms of their ability to undergo directed differentiation *in vitro* into cells representative of mesoderm, endoderm and ectoderm germinal layers. Directed differentiation was conducted according to protocols described previously [11-13]. To induce endo-mesoderm differentiation, pESC colonies were initially cultured in porcine ESC medium with an increased concentration of Activin A (100 ng/ml; R&D Systems) for 6-7 days.

For endoderm differentiation, endo-mesoderm differentiation medium was replaced with RPMI 1640 Medium (Life technologies, USA) with low (11 mM) glucose concentration

supplemented with 2% SR (Life technologies, USA), 10 mM nicotinamide, 10 nM exendin 4 (Abbotec, USA) and 50 ng/ml insulin growth factor II (IGF-II; Life technologies, USA) for another 15-25 days of culture. A few days before fixation the glucose concentration in medium was increased to 25 mM when the cells started to cluster. After culture, colonies were fixed and permeabilized as described above to perform Immunofluorescence for pancreatic and duodenal homeobox 1 (PDX-1). The primary antibody was rabbit anti-PDX-1 (1:500 dilution; Merk-Millipore, Germany) and the secondary antibody was donkey anti-rabbit IgG-Alexa 546 (1:200 dilution; A11055, Invitrogen). The F-actin of cell membranes was stained with Alexa Fluor-488 Phalloidin (Life technologies, USA). Fluorescent images were captured using Leica TCS SP5 confocal laser scanning microscope (Leica Microsystem, Germany).

For mesoderm differentiation into adipocytes, pESCs were also cultured with high concentration of activin A as described above. pESCs were then cultured in a 3–5 times rotation of adipogenic induction (AI) medium for 3 days, then adipogenic maintenance (AM) medium for 1 day. AI medium consisted of DMEM medium (Life technologies, USA) supplemented with 10% FBS, 0.6% antibiotic-antimycotic solution, 0.5 mM methyl-isobutylxanthine (IBMX), 100 mM indomethacin, 1 mM dexamethasone and 10 µg/ml insulin. AM medium consisted of DMEM medium (Life technologies, USA) supplemented with 10% FBS and 10 µg/ml insulin. Colonies were then maintained in AM medium for 4–7 days prior to fixation. Colonies were fixed in 4% paraformaldehyde and stained with Oil Red O. Nuclei were then counterstained with haematoxylin. The colonies were analyzed using Nikon Eclipse TS100 inverted microscope (Nikon Instruments Inc., Japan).

For ectoderm differentiation, pESCs were differentiated into neural-like cells by culturing in neural differentiation medium, which consisted of KnockOut™ DMEM/F12 supplemented with 2% SR, 1% N2 supplement, 55 mM 2-mercaptoethanol and 10 ng/ml bFGF (all from Life technologies, USA) for 30 days. pESC colonies were then fixed and permeabilised as described for immunofluorescence analysis above. Expression of nestin was used as an indicator of neural differentiation. The primary

antibody was goat anti-nestin IgG (1:200 dilution; Santa Cruz Biotech, USA) and secondary antibody was donkey anti-goat IgG-Alexa Fluor® 647 (1:200 dilution; Life technologies, USA). Fluorescence image analysis was performed using Leica TCS SP5 Confocal Microscope (Leica Microsystem, Wetzlar, Germany).

Results and discussion

The present study was undertaken to determine the efficiency with which porcine heterozygous parthenotes could be produced using CB and CD and the efficiency with which heterozygous pESCs could be isolated from these. To confirm heterozygosity previous researchers [5,9] have analyzed parthenogenetic embryos obtained from hybrid females of two mice strains with distinguishable protein isoforms or single nucleotide polymorphism profiles and compared these with their parents. In contrast our study used abattoir derived oocytes from predominately Large White x Landrace sows which also contain infusions of Duroc. This unknown mixed parentage did not allow us to determine which alleles are maternal or paternal in origin or the level of heterozygosity using single nucleotide polymorphism array or microsatellite analysis. Nevertheless having demonstrated the concept in mice, it is reasonable to assume that the suppression of the first polar body extrusion in porcine oocytes also results in the production of heterozygous parthenotes.

Effect of cytochalasin B and D on the production of heterozygous parthenotes and the isolation of pESCs

The effect of CB and CD on the development of heterozygous porcine parthenotes and the efficiency with which pESC lines could be derived from these is shown in (Table 1). A total 816 oocytes were treated with CB and 872 oocytes treated with CD. Cleavage rate was significantly lower in CB group compared with CD group (58.6 vs. 88.4%; $p < 0.001$). In both groups, oocytes were able to develop to the blastocysts stage at day 7 of culture (Figure 2A). However blastocyst development rate was significantly lower in CB group compared with the CD group (39.5 vs. 47.2%; $p < 0.01$).

Blastocysts in both groups were able to form homogeneous

Table 1. The effect of cytochalasin B and D on the production of heterozygous parthenotes and the isolation of parthenogenetic ESCs.

Chemicals	Activated oocytes (%)	Cleaved oocytes (%)	Blastocysts produced and plated (%)	Primary ESC outgrowths (%)	Primary ESC lines (%)	Established ESC lines (%)
Cytochalasin B	816	478 (58.6) ^a	189 (39.5) ^a	5 (2.6) ^a	0	0
Cytochalasin D	872	771 (88.4) ^c	364 (47.2) ^b	39 (10.7) ^c	8 (2.2)	2 (0.5)

Values with different superscripts within a column are significantly different. a and b different significantly with P value <0.01: a and c different significantly with P value <0.001.

primary embryonal outgrowths. However embryonal out growth rate was lower in in the CB group compared with the CD group ($p < 0.001$). Only five (2.65%) primary outgrowths were

established in CB group after plating 189 blastocysts, whereas in CD group 39 (10.7%) of 364 plated blastocysts were able to form primary outgrowths (Figure 2B) in both groups

embryonal outgrowths were morphologically homogeneous consisting entirely of pESCs. None of the outgrowths in CB groups survived to passage two to form primary cell lines. In the CD group eight parthenotes gave rise to the primary cell lines (2.2%) and two of these (0.5%) survived vitrification/warming resulting in established heterozygous pESC lines. pESCs had characteristic ESC morphology (Figure 2C). As described previously by us for ESCs isolated from *in vitro* produced and somatic cell nuclear transfer (SCNT) embryos [11,13] namely ESCs were relatively small in size (10–15 µm in diameter) and polygonal in shape. The nucleus occupied practically all of the volume of the cell and held multiple nucleoli. Cells also have numerous lipid inclusions in the cytoplasm.

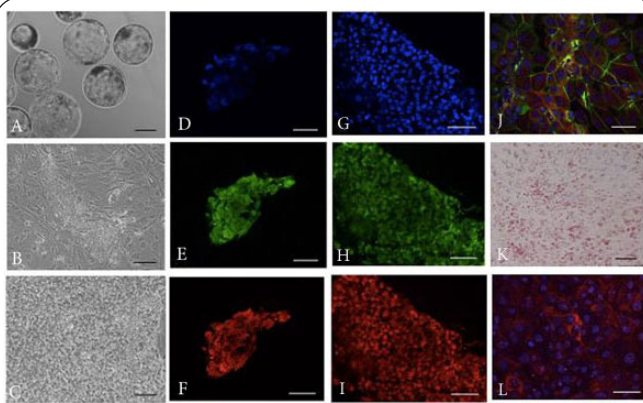


Figure 2. Production of porcine heterozygous parthenotes and characterization of ESC lines. (A) Porcine heterozygous parthenotes produced after treatment of oocytes with cytochalasin D. (B) Morphologically homogeneous primary embryonal outgrowth from heterozygous parthenote produced using cytochalasin D 5 days after plating. (C) Established porcine pESCs at passage 5 isolated from heterozygous parthenotes produced using cytochalasin D. Primary embryonal outgrowths (D-E) and established heterozygous pESCs (G-I) co-expressed the pluripotency markers Oct-4 (green) and Nanog (red). Nuclei are stained with blue (DAPI). Heterozygous pESCs could be directly differentiated into representative of all three germ layers. Analysis of endoderm differentiation (J) demonstrated that under differentiation conditions pdx-1 (red) was found in cytoplasm and in the nuclei (small red dots in nuclei) of differentiating cells indicating the migration of pdx-1 from cytoplasm into the nucleus (blue) in response to changing glucose concentrations. Cell membranes stained green. Mesodermal differentiation (K) resulted in formation of adipocytes with fat droplets stained with red. Ectodermal differentiation (L) resulted in the formation of nestin-positive cells (red). Bar in Fig. 2K: 50 µm. Bars in all other images: 100µm

Characterization of heterogeneous parthenogenetic ESC lines

Immunofluorescence analysis of one of the primary embryonal outgrowths and established cell line isolated from it at passage 5 demonstrated that all cells of outgrowth (Figures 2D and 2F)

and cell line (Figures 2G and 2I) co-expressed of the pluripotent markers Oct4 and Nanog. Established heterozygous pESC lines were maintained in culture until passage eight and then were successfully differentiated into cell types representative of all three germ layers. Endoderm lineage differentiation of heterozygous ESC into pancreatic islet progenitor cells resulted in appearance of cells expressing pdx-1-protein that is involved in the early development of the pancreas and plays a major role in glucose-dependent regulation of insulin gene expression. Although pdx 1 is an insulin transcription factor, its localization in cells is glucose-dependent. When cells are cultured in media containing low glucose pdx1 is localized in cytoplasm, whereas following an increase in glucose concentration pdx1 migrates to the nucleus [18]. In our experiments endoderm differentiation was induced in medium containing low glucose to promote cell expansion. These cells were then cultured in high glucose for a few days immediately prior to fixation resulting in the presence of pdx-1 protein in both compartments as described previously by us for ESCs isolated from *in vitro* produced and somatic cell nuclear transfer embryos [11,13].

As result pdx-1 protein was detected both in cytoplasm and nuclei (Figure 2J), and this testifies the beginning of the migration of pdx-1 into nucleus as expected. Culture in mesodermal lineage differentiation medium containing adipogenic factors produced adipocytes (Figure 2K) as confirmed with Oil Red O staining. Culture of ESCs in neural differentiation medium for 30 days produced nestin-positive neural precursor cells, which had diffusive cytoplasmic staining (Figure 2L). Besides its cytoplasmic localization, nestin also was found in nuclei. This is not surprising because ESC is vulnerable to neoplastic formation and during neuronal differentiation can form cells, which share properties with neural neoplastic cells. Such cells can exhibit both nuclear and nucleolar localization of nestin [19] which can interact with DNA [20].

The present study was undertaken to determine the efficiency with which porcine heterozygous parthenotes could be produced using cytochalasin B and D and the efficiency with which heterozygous pESCs could be isolated from these. CB is commonly used for SCNT to relax the cytoskeleton and prevent the extrusion of transferred nuclei in enucleated oocytes. In contrast, CD is rarely used for this purpose. However, treatment of SCNT embryos with CB results in a significant reduction in cleavage and blastocyst rates compared with CD [21]. Similar effects were seen in our study when CB was used to produce diploidized heterozygous parthenotes. Treating oocytes for 22h with CB resulted in significantly lower cleavage and blastocyst formation rates compared with that for the CD treated group. Blastocysts from CB treated group also formed primary ESC outgrowths at a significantly lower level than those from the CD treated group. Furthermore, no primary or established putative pESC lines were obtained from outgrowths in the CB group. In contrast, eight primary

and two established cell lines were isolated in the CD group. Established pESC lines had a characteristic ESC morphology and expressed Oct-4 and Nanog following repeated passaging. Furthermore, these cell lines could be directly differentiated to cell types representatives of all three germ layers.

The failure to establish cell lines from heterozygous parthenotes using CB could be explained by its known cytotoxic effect in particular its ability to inhibit glucose transport across the plasma membrane not only in the maturing oocyte but also in the surrounding cumulus cells. It is known that cumulus cells are very important both for maintenance of developmental potential of porcine oocytes and for distribution and functionality of cortical granules and mitochondria [22]. The cytotoxic effect of CB on cumulus cells may affect the cytoplasmic maturation of oocytes and developmental competence of pluripotent cells of parthenotes. In contrast, CD has been shown not to interfere with glucose transport [10]. In addition, CD treated parthenogenetic oocytes show faster reorganization of cortical F-actin after artificial activation, which is critical for normal mitochondria distribution, and the transfer of these to the daughter cells during cell division compared with CB treatment [21]. However, previous attempts in our laboratory to isolate ESCs from pig homozygous parthenotes using CD have also failed (unpublished results). This suggests that level of heterozygosity may also affect the derivation of pESC lines possibly through the expression of recessive lethal and sublethal genes as well as the aberrant expression of imprinted genes [23]. This is consistent with previous attempts to isolate pESCs from pig homozygous parthenotes produced using ionomycin and 6-DMAP [24] or electrical activation and CB treatment [25] where isolated pESCs had limited developmental potential and a relatively short lifespan in culture. As such, we suggest that the cytotoxicity of diploidization agent and the degree of heterozygosity are important factors in the isolation of parthenogenetic ESCs.

In conclusion, we have demonstrated that CD can be used to generate heterozygous parthenotes from which putative parthenogenetic ESC lines can be established. These cells had a characteristic ESC morphology and could be differentiated to cell types representative of all three germ layers. This is the first time to our knowledge that putative ESC has been isolated from porcine heterozygous parthenotes. In contrast, ESCs could not be isolated using CB possibly because of its increased toxic effects as a consequence of the relatively long exposure time required to produce heterozygous parthenotes. Further work is required to confirm the pluripotency of these pESCs. In particular, further *in vivo* characterization of these cells is required including teratoma formation in immune compromised mice, chimaera production and the demonstration of germline transmission as has been demonstrated previously for homozygous mouse pESCs [6,7].

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	IV	AT	WXK	SM	MBN
Research concept and design	✓	--	--	--	✓
Collection and/or assembly of data	✓	✓	✓	✓	--
Data analysis and interpretation	✓	✓	✓	--	✓
Writing the article	✓	✓	✓	--	✓
Critical revision of the article	✓	--	--	--	✓
Final approval of article	✓	--	--	--	✓
Statistical analysis	✓	✓	✓	--	✓

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